



(86) Date de dépôt PCT/PCT Filing Date: 2000/01/29
(87) Date publication PCT/PCT Publication Date: 2000/08/03
(45) Date de délivrance/Issue Date: 2007/06/12
(85) Entrée phase nationale/National Entry: 2001/07/18
(86) N° demande PCT/PCT Application No.: DE 2000/000244
(87) N° publication PCT/PCT Publication No.: 2000/044895
(30) Priorités/Priorities: 1999/01/30 (DE199 03 713.2);
1999/11/24 (DE199 56 568.6)

(51) Cl.Int./Int.Cl. *C12N 15/11* (2006.01),
A61K 31/713 (2006.01), *A61P 31/12* (2006.01),
C07H 21/02 (2006.01), *C12N 5/06* (2006.01),
C12N 5/08 (2006.01), *A61K 38/00* (2006.01)
(72) Inventeurs/Inventors:
KREUTZER, ROLAND, DE;
LIMMER, STEPHAN, DE
(73) Propriétaire/Owner:
ALNYLAM EUROPE AG, DE
(74) Agent: SMART & BIGGAR

(54) Titre : METHODE ET MEDICAMENT DESTINES A INHIBER L'EXPRESSION D'UN GENE DONNE
(54) Title: METHOD AND MEDICAMENT FOR INHIBITING THE EXPRESSION OF A GIVEN GENE

(57) Abrégé/Abstract:

The invention relates to a medicament containing at least one double-stranded oligoribonucleotide (dsRNA) designed to inhibit the expression of a target gene. According to the invention, one strand of the dsRNA is at least in part complementary to the target gene.





PCT
WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro
**INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE
INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)**

<p>(51) Internationale Patentklassifikation ⁷ : C12N 15/11, A61K 31/713</p>	<p>A1</p>	<p>(11) Internationale Veröffentlichungsnummer: WO 00/44895</p> <p>(43) Internationales Veröffentlichungsdatum: 3. August 2000 (03.08.00)</p>
<p>(21) Internationales Aktenzeichen: PCT/DE00/00244</p> <p>(22) Internationales Anmeldedatum: 29. Januar 2000 (29.01.00)</p> <p>(30) Prioritätsdaten: 199 03 713.2 30. Januar 1999 (30.01.99) DE 199 56 568.6 24. November 1999 (24.11.99) DE</p> <p>(71)(72) Anmelder und Erfinder: KREUTZER, Roland [DE/DE]; Glotzdorf 26, D-95466 Weidenberg (DE). LIMMER, Stephan [DE/DE]; Leibnizstrasse 14, D-95447 Bayreuth (DE).</p> <p>(74) Anwalt: GASSNER, Wolfgang; Nägelsbachstrasse 49 A, D-91052 Erlangen (DE).</p>	<p>(81) Bestimmungsstaaten: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Veröffentlicht <i>Mit internationalem Recherchenbericht. Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist; Veröffentlichung wird wiederholt falls Änderungen eintreffen.</i></p>	
<p>(54) Title: METHOD AND MEDICAMENT FOR INHIBITING THE EXPRESSION OF A DEFINED GENE</p> <p>(54) Bezeichnung: VERFAHREN UND MEDIKAMENT ZUR HEMMUNG DER EXPRESSION EINES VORGEGEBENEN GENS</p> <p>(57) Abstract The invention relates to a medicament containing at least one double-stranded oligoribonucleotide (dsRNA) designed to inhibit the expression of a target gene. According to the invention, one strand of the dsRNA is at least in part complementary to the target gene.</p> <p>(57) Zusammenfassung Die Erfindung betrifft ein Medikament mit mindestens einem Oligoribonukleotid mit doppelsträngiger Struktur (dsRNA) zur Hemmung der Expression eines Zielgens, wobei ein Strang der dsRNA zumindest abschnittsweise komplementär zum Zielgen ist.</p>		

WO 00/44895

PCT/DE00/00244

Method and medicament for inhibiting the expression of a given gene

5 The invention relates to methods in accordance with the preambles of claims 1 and 2. It furthermore relates to a medicament and to a use of double-stranded oligoribonucleotides and to a vector encoding them.

10 Such a method is known from WO 99/32619, which was unpublished at the priority date of the present invention. The known process aims at inhibiting the expression of genes in cells of invertebrates. To this end, the double-stranded oligoribonucleotide must exhibit a sequence which is identical with the target
15 gene and which has a length of at least 50 bases. To achieve efficient inhibition, the identical sequence must be 300 to 1 000 base pairs in length. Such an oligoribonucleotide is complicated to prepare.

20 DE 196 31 919 C2 describes an antisense RNA with specific secondary structures, the antisense RNA being present in the form of a vector encoding it. The antisense RNA takes the form of an RNA molecule which is complementary to regions of the mRNA. Inhibition of
25 the gene expression is caused by binding to these regions. This inhibition can be employed in particular for the diagnosis and/or therapy of diseases, for example tumor diseases or viral infections. - The disadvantage is that the antisense RNA must be
30 introduced into the cell in an amount which is at least as high as the amount of the mRNA. The known antisense methods are not particularly effective.

US 5,712,257 discloses a medicament comprising
35 mismatched double-stranded RNA (dsRNA) and bioactive mismatched fragments of dsRNA in the form of a ternary complex together with a surfactant. The dsRNA used for this purpose consists of synthetic nucleic acid single strands without defined base sequence. The single

29824-21

- 2 -

strands undergo irregular base pairing, also known as "non-Watson-Crick" base pairing, giving rise to mismatched double strands. The known dsRNA is used to inhibit the amplification of retroviruses such as HIV.

- 5 Amplification of the virus can be inhibited when non-sequence-specific dsRNA is introduced into the cells. This leads to the induction of interferon, which is intended to inhibit viral amplification. The inhibitory effect, or the activity, of this method is poor.

10

It is known from Fire, A. et al., NATURE, Vol. 391, pp. 806 that dsRNA whose one strand is complementary in segments to a nematode gene to be inhibited inhibits the expression of this gene highly efficiently. It is

- 15 believed that the particular activity of the dsRNA used in nematode cells is not due to the antisense principle but possibly on catalytic properties of the dsRNA, or enzymes induced by it. - Nothing is mentioned in this paper on the activity of specific dsRNA with regard to
20 inhibiting the gene expression, in particular in mammalian and human cells.

- The object of the present invention is to do away with the disadvantages of the prior art. In particular, it
25 is intended to provide as effective as possible a method, medicament or use for the preparation of a medicament, which method, medicament or use is capable of causing particularly effective inhibition of the expression of a given target gene.

29824-21

- 3 -

Provided in accordance with the invention are an oligoribonucleotide or a vector encoding therefor. At least segments of the oligoribonucleotide exhibit a defined nucleotide sequence. The defined segment may be limited to the complementary region I. However, it is also possible that all of the double-stranded oligoribonucleotide exhibits a defined nucleotide sequence.

Surprisingly, it has emerged that an effective inhibition of the expression of the target gene can be achieved even when the complementary region I is not more than 49 base pairs in length. The procedure of providing such oligoribonucleotides is less complicated.

In particular, dsRNA with a length of over 50 nucleotide pairs induces certain cellular mechanisms, for example the dsRNA-dependent protein kinase or the 2-5A system, in mammalian and human cells. This leads to the disappearance of the interference effect mediated by the dsRNA which exhibits a defined sequence. As a consequence, protein biosynthesis in the cell is blocked. The present invention overcomes this disadvantage in particular.

Furthermore, the uptake of dsRNA with short chain lengths into the cell or into the nucleus is facilitated markedly over longer-chain dsRNAs.

It has proved advantageous for the dsRNA or the vector to be present packaged into micellar structures, preferably in liposomes. The dsRNA or the vector can likewise be enclosed in viral natural capsids or in chemically or enzymatically produced artificial capsids or structures derived therefrom. - The abovementioned features make it possible to introduce the dsRNA or the vector into given target cells.

- 4 -

In a further aspect, the dsRNA has 10 to 1 000, preferably 15 to 49, base pairs. Thus, the dsRNA can be longer than the region I, which is complementary to the target gene. The complementary region I can be located
5 at the terminus or inserted into the dsRNA. Such dsRNA or a vector provided for coding the same can be produced synthetically or enzymatically by customary methods.

10 The gene to be inhibited is expediently expressed in eukaryotic cells. The target gene can be selected from the following group: oncogene, cytokin gene, Id protein gene, developmental gene, prion gene. It can also be expressed in pathogenic organisms, preferably in
15 plasmodia. It can be part of a virus or viroid which is preferably pathogenic to humans. - The method proposed makes it possible to produce compositions for the therapy of genetically determined diseases, for example cancer, viral diseases or Alzheimer's disease.

20 The virus or viroid can also be a virus or viroid which is pathogenic to animals or plant-pathogenic. In this case, the method according to the invention also permits the provision of compositions for treating
25 animal or plant diseases.

In a further aspect, segments of the dsRNA are designed as double-stranded. A region II which is complementary within the double-stranded structure is formed by two
30 separate RNA single strands or by autocomplementary regions of a topologically closed RNA single strand which is preferably in circular form.

The ends of the dsRNA can be modified to counteract
35 degradation in the cell or dissociation into the single strands. Dissociation takes place in particular when low concentrations or short chain lengths are used. To inhibit dissociation in a particularly effective fashion, the cohesion of the complementary region II,

- 5 -

which is caused by the nucleotide pairs, can be increased by at least one, preferably two, further chemical linkage(s). - A dsRNA according to the invention whose dissociation is reduced exhibits
5 greater stability to enzymatic and chemical degradation in the cell or in the organism.

The complementary region II can be formed by autocomplementary regions of an RNA hairpin loop, in
10 particular when using a vector according to the invention. To afford protection from degradation, it is expedient for the nucleotides to be chemically modified in the loop region between the double-stranded structure.

15 The chemical linkage is expediently formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, preferably van-der-Waals or stacking interactions, or by metal-ion coordination. In an
20 especially advantageous aspect, it can be formed at at least one, preferably both, end(s) of the complementary region II.

It has furthermore proved to be advantageous for the
25 chemical linkage to be formed by one or more linkage groups, the linkage groups preferably being poly(oxyphosphinicooxy-1,3-propanediol) and/or polyethylene glycol chains. The chemical linkage can also be formed by purine analogs used in place of purines in
30 the complementary regions II. It is also advantageous for the chemical linkage to be formed by azabenzene units introduced into the complementary regions II. Moreover, it can be formed by branched nucleotide analogs used in place of nucleotides in the
35 complementary regions II.

It has proved expedient to use at least one of the following groups for generating the chemical linkage: methylene blue; bifunctional groups, preferably

29824-21(S)

- 6 -

bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxyl-benzoyl)cystamine; 4-thiouracil; psoralene. The chemical linkage can furthermore be formed by thiophosphoryl groups provided at the ends of the double-stranded region. The chemical linkage at the ends of the double-stranded region is preferably formed by triple-helix bonds.

The chemical linkage can expediently be induced by ultraviolet light.

The nucleotides of the dsRNA can be modified. This counteracts the activation, in the cell, of a double-stranded-RNA-dependent protein kinase, PKR. Advantageously, at least one 2'-hydroxyl group of the nucleotides of the dsRNA in the complementary region II is replaced by a chemical group, preferably a 2'-amino or a 2'-methyl group. At least one nucleotide in at least one strand of the complementary region II can also be a locked nucleotide with a sugar ring which is chemically modified, preferably by a 2'-O, 4'-C methylene bridge. Advantageously, several nucleotides are locked nucleotides.

A further especially advantageous embodiment provides that the dsRNA or the vector is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically. The coat protein can be derived from polyomavirus. The coat protein can contain the polyomavirus virus protein 1 (VP1) and/or virus protein 2 (VP2). The use of such coat proteins is known from, for example, DE 196 18 797 A1. - The abovementioned features considerably facilitate the introduction of the dsRNA or of the vector into the cell.

52032-2(S)

- 7 -

When a capsid or capsid-type structure is formed from the coat protein, one side preferably faces the interior of the capsid or capsid-type structure. The construct formed is particularly stable.

5 The dsRNA can be complementary to the primary or processed RNA transcript of the target gene. The cell can be a vertebrate cell or a human cell.

 At least two dsRNAs which differ from each other or at least one vector encoding them can be introduced into
10 the cell, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes. This makes it possible simultaneously to inhibit the expression of at least two different target genes. In order to suppress, in the cell,
15 the expression of a double-stranded-RNA-dependent protein kinase, PKR, one of the target genes is advantageously the PKR gene. This allows effective suppression of the PKR activity in the cell.

 The invention furthermore provides a medicament
20 with at least one oligoribonucleotide with double-stranded structure (dsRNA) for inhibiting the expression of a given target gene, where one strand of the dsRNA has a region I where at least segments are complementary to the target gene. Surprisingly, it has emerged that such a dsRNA is
25 suitable as medicament for inhibiting the expression of a given gene in mammalian cells. In comparison with the use of single-stranded oligoribonucleotides, the inhibition is already caused at concentrations which are lower by at least one order of magnitude. The medicament according to the
30 invention is highly effective. Lesser side effects can be expected.

52032-2(S)

- 8 -

The invention furthermore provides a medicament with at least one vector for coding at least one oligoribonucleotide with double-stranded structure (dsRNA) for inhibiting the expression of a given target gene, where
5 one strand of the dsRNA has a region I where at least segments are complementary to the target gene. The medicament proposed exhibits the abovementioned advantages. By using a vector, in particular production costs can be reduced.

10 In a particularly advantageous embodiment, the complementary region I has not more than 49 successive nucleotide pairs. Surprisingly, it has emerged that an effective inhibition of the expression of the target gene can be achieved even when the complementary region I is not
15 more than 49 base pairs in length. The procedure of providing such oligoribonucleotides is less complicated.

The invention furthermore provides a use of an oligoribonucleotide with double-stranded structure (dsRNA) for preparing a medicament for inhibiting the expression of
20 a given target gene, where one strand of the dsRNA has a region I where at least segments are complementary to the target gene. Surprisingly, such a dsRNA is suitable for preparing a medicament for inhibiting the expression of a given gene. Compared with the use of single-stranded
25 oligoribonucleotides, the inhibition is already caused at concentrations which are lower by one order of magnitude when using dsRNA. The use according to the invention thus makes possible the preparation of particularly effective medicaments.

30 The invention furthermore provides the use of a vector for coding at least one oligoribonucleotide with double-stranded structure (dsRNA) for preparing a medicament

52032-2(S)

- 8a -

for inhibiting the expression of a given target gene, where one strand of the dsRNA has a region I where at least segments are complementary to this target gene. The use of a vector makes possible a particularly effective gene
5 therapy.

In another aspect, the invention provides a method for inhibiting the expression of a given target gene in a mammalian cell, the method comprising introducing into the mammalian cell an oligoribonucleotide with double-stranded
10 structure (dsRNA) formed by two separate RNA single strands, said dsRNA having a length of 15 to 49 base pairs, wherein one of said RNA single strands has a region which is at least partially complementary to a given target gene expressed in a mammalian cell, wherein said method is not
15 practised on the human or animal body.

In another aspect, the invention provides medicament for inhibiting the expression of a given target gene in mammalian cells, said medicament comprising an oligoribonucleotide with double-stranded structure (dsRNA)
20 formed by two separate RNA single strands, said dsRNA having a length of 15 to 49 base pairs, wherein one of said RNA single strands has a region which is at least partially complementary to a given target gene expressed in a mammalian cell.

25 In another aspect, the invention provides use, for inhibiting the expression of a given target gene in mammalian cells, of an oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands, said dsRNA having a length of 15 to 49 base pairs,
30 wherein one of said RNA single strands has a region which is at least partially complementary to a given target gene expressed in a mammalian cell.

52032-2(S)

- 8b -

In another aspect, the invention provides an oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands, said dsRNA having a length of 15 to 49 base pairs, wherein one of said RNA
5 single strands has a region which is at least partially complementary to a given target gene expressed in a mammalian cell.

In another aspect, the invention provides a composition comprising an oligoribonucleotide as described
10 above, and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a mammalian cell comprising an exogenous oligoribonucleotide, wherein the exogenous oligoribonucleotide is an oligoribonucleotide as described above.

29824-21

- 9 -

With regard to advantageous embodiments of the medicament and of the use, reference is made to the description of the above features.

5

Use examples of the invention are illustrated in greater detail hereinbelow with reference to the figures, in which:

10 Fig. 1 shows the schematic representation of a plasmid for the *in vitro* transcription with T7- and SP6-polymerase,

15 Fig. 2 shows RNA following electrophoresis on an 8% polyacrylamide gel and staining with ethidium bromide,

20 Fig. 3 shows a representation of radioactive RNA transcripts following electrophoresis on an 8% polyacrylamide gel with 7 M urea by means of an instant imager, and

Figs. 4a - e show Texas Red and YFP fluorescence in murine fibroblasts.

25

Use example 1:

The inhibition of transcription was detected by means of sequence homologous dsRNA in an *in vitro* transcription system with a nuclear extract from human
30 HeLa cells. The DNA template for this experiment was plasmid pCMV1200 which had been linearized by means of *Bam*HI.

Generation of the template plasmids:

35 The plasmid shown in fig. 1 was constructed for use in the enzymatic synthesis of the dsRNA. To this end, a polymerase chain reaction (PCR) with the "positive control DNA" of the HelaScribe® Nuclear Extract *in vitro* transcription kit by Promega, Madison, USA, as

- 10 -

DNA template was first carried out. One of the primers used contained the sequence of an *EcoRI* cleavage site and of the T7 RNA polymerase promoter as shown in sequence listing No. 1. The other primer contained the sequence of a *BamHI* cleavage site and of the SP6 RNA polymerase promoter as shown in sequence listing No. 2. In addition, the two primers had, at the 3' ends, regions which were identical with or complementary to the DNA template. The PCR was carried out by means of the "Taq PCR Core Kits" by Qiagen, Hilden, Germany, following the manufacturer's instructions. 1.5 mM MgCl₂, in each case 200 μM dNTP, in each case 0.5 μM primer, 2.5 U Taq DNA polymerase and approximately 100 ng of "positive control DNA" were employed as template in PCR buffer in a volume of 100 μl. After initial denaturation of the template DNA by heating for 5 minutes at 94°C, amplification was carried out in 30 cycles of denaturation for in each case 60 seconds at 94°C, annealing for 60 seconds at 5°C below the calculated melting point of the primers and polymerization for 1.5-2 minutes at 72°C. After a final polymerization of 5 minutes at 72°C, 5 μl of the reaction were analyzed by agarose-gel electrophoresis. The length of the DNA fragment amplified thus was 400 base pairs, 340 base pairs corresponding to the "positive control DNA". The PCR product was purified, hydrolyzed with *EcoRI* and *BamHI* and, after repurification, employed in the ligation together with a pUC18 vector which had also been hydrolyzed by *EcoRI* and *BamHI*. *E. coli* XL1-blue was then transformed. The plasmid obtained (pCMV5) carries a DNA fragment whose 5' end is flanked by the T7 promoter and whose 3' end is flanked by the SP6 promoter. By linearizing the plasmid with *BamHI*, it can be employed *in vitro* with the T7-RNA polymerase for the run-off transcription of a single-stranded RNA which is 340 nucleotides in length and shown in sequence listing No. 3. If the plasmid is linearized with *EcoRI*, it can be employed for the run-off transcription with SP6 RNA polymerase,

- 11 -

giving rise to the complementary strand. In accordance with the method outlined hereinabove, an RNA 23 nucleotides in length was also synthesized. To this end, a DNA shown in sequence listing No. 4 was ligated
5 with the pUC18 vector via the *EcoRI* and *BamHI* cleavage sites.

Plasmid pCMV1200 was constructed as DNA template for the *in-vitro* transcription with HeLa nuclear extract.
10 To this end, a 1 191 bp *EcoRI*/*BamHI* fragment of the positive control DNA contained in the HeLaScribe® Nuclear Extract *in vitro* transcription kit was amplified by means of PCR. The amplified fragment encompasses the 828 bp "immediate early" CMV promoter
15 and a 363 bp transcribable DNA fragment. The PCR product was ligated to the vector pGEM-T via "T-overhang" ligation. A *BamHI* cleavage site is located at the 5' end of the fragment. The plasmid was linearized by hydrolysis with *BamHI* and used as
20 template in the run-off transcription.

In-vitro transcription of the complementary single strands:

pCMV5 plasmid DNA was linearized with *EcoRI* or *BamHI*.
25 It was used as DNA template for an *in-vitro* transcription of the complementary RNA single strands with SP6 and T7 RNA polymerase, respectively. The "Riboprobe *in vitro* Transcription" system by Promega, Madison, USA, was employed for this purpose. Following
30 the manufacturer's instructions, 2 µg of linearized plasmid DNA were incubated in 100 µl of transcription buffer and 40 U T7 or SP6 RNA polymerase for 5-6 hours at 37°C. The DNA template was subsequently degraded by addition of 2.5 µl of RNase-free DNase RQ1 and
35 incubation for 30 minutes at 37°C. The transcription reaction was made up to 300 µl with H₂O and purified by phenol extraction. The RNA was precipitated by addition of 150 µl of 7 M ammonium acetate [sic] and 1 125 µl of

- 12 -

ethanol and stored at -65°C until used for the hybridization.

Generation of the RNA double strands:

- 5 For the hybridization, $500\text{ }\mu\text{l}$ of the single-stranded RNA which had been stored in ethanol and precipitated were spun down. The resulting pellet was dried and taken up in $30\text{ }\mu\text{l}$ of PIPES buffer, pH 6.4 in the presence of 80% formamide, 400 mM NaCl and 1 mM EDTA.
- 10 In each case $15\text{ }\mu\text{l}$ of the complementary single strands were combined and heated for 10 minutes at 85°C . The reactions were subsequently incubated overnight at 50°C and cooled to room temperature.
- 15 Only approximately equimolar amounts of the two single strands were employed in the hybridization. This is why the dsRNA preparations contained single-stranded RNA (ssRNA) as contaminant. In order to remove these ssRNA contaminants, the reactions were treated, after
- 20 hybridization, with the single-strand-specific ribonucleases bovine pancreatic RNase A and *Aspergillus oryzae* RNase T1. RNase A is an endoribonuclease which is specific for pyrimidines. RNase T1 is an endoribonuclease which preferentially cleaves at the 3'
- 25 side of guanosines. dsRNA is no substrate for these ribonucleases. For the RNase treatment, the reactions in $300\text{ }\mu\text{l}$ of Tris, pH 7.4, 300 mM NaCl and 5 mM EDTA were treated with $1.2\text{ }\mu\text{l}$ of RNaseA at a concentration of 10 mg/ml and $2\text{ }\mu\text{l}$ of RNaseT1 at a concentration of
- 30 $290\text{ }\mu\text{g/ml}$. The reactions were incubated for 1.5 hours at 30°C . Thereupon, the RNases were denatured by addition of $5\text{ }\mu\text{l}$ of proteinase K at a concentration of 20 mg/ml and $10\text{ }\mu\text{l}$ of 20% SDS and incubation for 30 minutes at 37°C . The dsRNA was purified by phenol
- 35 extraction and precipitated with ethanol. To verify the completeness of the RNase digestion, two control reactions were treated with ssRNA analogously to the hybridization reactions.

- 13 -

The dried pellet was taken up in 15 μ l of TE buffer, pH 6.5, and subjected to native polyacrylamide gel electrophoresis on an 8% gel. The acrylamide gel was subsequently stained in an ethidium bromide solution and washed in a water bath. Fig. 2 shows the RNA which had been visualized in a UV transilluminator. The sense RNA which had been applied to lane 1 and the antisense RNA which had been applied to lane 2 showed a different migration behavior under the chosen conditions than the dsRNA of the hybridization reaction which had been applied to lane 3. The RNase-treated sense RNA and antisense RNA which had been applied to lanes 4 and 5, respectively, produced no visible band. This shows that the single-stranded RNAs had been degraded completely. The RNase-treated dsRNA of the hybridization reaction which had been applied to lane 6 is resistant to RNase treatment. The band which migrates faster in the native gel in comparison with the dsRNA applied to lane 3 results from dsRNA which is free from ssRNA. In addition to the dominant main band, weaker bands which migrate faster are observed after the RNase treatment.

In-vitro transcription test with human nuclear extract:

Using the HeLaScribe[®] Nuclear Extract *in vitro* transcription kit by Promega, Madison, USA, the transcription efficiency of the abovementioned DNA fragment which is present in plasmid pCMV1200 and homologous to the "positive control DNA" was determined in the presence of the dsRNA (dsRNA-CMV5) with sequence homology. Also, the effect of the dsRNA without sequence homology, which corresponds to the yellow fluorescent protein (YFP) gene (dsRNA-YRP), was studied. This dsRNA had been generated analogously to the dsRNA with sequence homology. The sequence of a strand of this dsRNA can be found in sequence listing No. 5. Plasmid pCMV1200 was used as template for the run-off transcription. It carries the "immediate early" cytomegalovirus promoter which is recognized by the eukaryotic RNA polymerase II, and a transcribable DNA

- 14 -

fragment. Transcription was carried out by means of the HeLa nuclear extract, which contains all the proteins which are necessary for transcription. By addition of [γ - 32 P]rGTP to the transcription reaction, radiolabeled

5 transcript was obtained. The [γ - 32 P]rGTP used had a specific activity of 400 Ci/mmol, 10 mCi/ml. 3 mM MgCl₂, in each case 400 μ M rATP, rCTP, rUTP, 16 μ M rGTP, 0.4 μ M [γ - 32 P]rGTP and depending on the experiment

10 1 fmol of linearized plasmid DNA and various amounts of dsRNA in transcription buffer were employed per reaction. Each batch was made up to a volume of 8.5 μ l with H₂O. The reactions were mixed carefully. To start the transcription, 4 U HeLa nuclear extract in a volume of 4 μ l were added and incubated for 60 minutes at

15 30°C. The reaction was stopped by addition of 87.5 μ l of quench mix which had been warmed to 30°C. To remove the proteins, the reactions were treated with 100 μ l of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) saturated with TE buffer, pH 5.0, and the reactions

20 were mixed vigorously for 1 minute. For phase separation, the reactions were spun for approximately 1 minute at 12 000 rpm and the top phase was transferred into a fresh reaction vessel. Each reaction was treated with 250 μ l of ethanol. The reactions were

25 mixed thoroughly and incubated for at least 15 minutes on dry ice/methanol. To precipitate the RNA, the reactions were spun for 20 minutes at 12 000 rpm and 40°C. The supernatant was discarded. The pellet was dried in vacuo for 15 minutes and resuspended in 10 μ l

30 of H₂O. Each reaction was treated with 10 μ l of denaturing loading buffer. The free GTP was separated from the transcript formed by means of denaturing polyacrylamide gel electrophoresis on an 8% gel with 7 M urea. The RNA transcripts formed upon transcription

35 with HeLa nuclear extract, in denaturing loading buffer, were heated for 10 minutes at 90°C and 10 μ l aliquots were applied immediately to the freshly washed pockets. The electrophoresis was run at 40 mA. The amount of the radioactive ssRNA formed upon

29824-21(S)

- 15 -

transcription was analyzed after electrophoresis with the aid of an *Instant Imager*.

Fig. 3 shows the radioactive RNA from a representative test, shown by means of the *Instant Imager**. Samples obtained from the following transcription reactions were applied:

- Lane 1: without template DNA, without dsRNA;
- 10 Lane 2: 50 ng of template DNA, without dsRNA;
- Lane 3: 50 ng of template DNA, 0.5 μ g of dsRNA YFP;
- Lane 4: 50 ng of template DNA, 1.5 μ g of dsRNA YFP;
- Lane 5: 50 ng of template DNA, 3 μ g of dsRNA YFP;
- Lane 6: 50 ng of template DNA, 5 μ g of dsRNA YFP;
- 15 Lane 7: without template DNA, 1.5 dsRNA YFP;
- Lane 8: 50 ng of template DNA, without dsRNA;
- Lane 9: 50 ng of template DNA, 0.5 μ g of dsRNA CMV5;
- Lane 10: 50 ng of template DNA, 1.5 μ g of dsRNA CMV5;
- Lane 11: 50 ng of template DNA, 3 μ g of dsRNA CMV5;
- 20 Lane 12: 50 ng of template DNA, 5 μ g of dsRNA CMV5;

It emerged that the amount of transcript was reduced markedly in the presence of dsRNA with sequence homology in comparison with the control reaction without dsRNA and with the reactions with dsRNA YFP without sequence homology. The positive control in lane 2 shows that radioactive transcript was formed upon the *in-vitro* transcription with HeLa nuclear extract. The reaction is used for comparison with the transcription reactions which had been incubated in the presence of dsRNA. Lanes 3 to 6 show that the addition of non-sequentially-specific dsRNA YFP had no effect on the amount of transcript formed. Lanes 9 to 12 show that the addition of an amount of between 1.5 and 3 μ g of sequentially-specific dsRNA CMV5 leads to a reduction in the amount of transcript formed. In order to exclude that the effects observed are based not on the dsRNA but on any contamination which might have been carried along accidentally during the preparation of the dsRNA,

35

*Trade-mark

- 16 -

a further control was carried out. Single-stranded RNA was transcribed as described above and subsequently subjected to the RNase treatment. It was demonstrated by means of native polyacrylamide gel electrophoresis that the ssRNA had been degraded completely. This reaction was subjected to phenol extraction and ethanol precipitation and subsequently taken up in PE buffer, as were the hybridization reactions. This gave a sample which contained no RNA but had been treated with the same enzymes and buffers as the dsRNA. Lane 8 shows that the addition of this sample had no effect on transcription. The reduction of the transcript upon addition of sequence-specific dsRNA can therefore be ascribed unequivocally to the dsRNA itself. The reduction of the amount of transcript of a gene in the presence of dsRNA in a human transcription system indicates an inhibition of the expression of the gene in question. This effect can be attributed to a novel mechanism caused by the dsRNA.

20

Use example 2:

The test system used for these *in-vivo* experiments was the murine fibroblast cell line NIH3T3, ATCC CRL-1658. The YFP gene was introduced into the nuclei with the aid of microinjection. Expression of YFP was studied under the effect of simultaneously cotransfected dsRNA with sequence homology. This dsRNA YFP shows homology with the 5'-region of the YFP gene over a length of 315 bp. The nucleotide sequence of a strand of the dsRNA YRP is shown in sequence listing No. 5. Evaluation under the fluorescence microscope was carried out 3 hours after injection with reference to the greenish-yellow fluorescence of the YFP formed.

35 **Construction of the template plasmid, and preparation of the dsRNA:**

A plasmid was constructed following the same principle as described in use example 1 to act as template for the production of the YFP dsRNA by means of T7 and SP6

- 17 -

in-vitro transcription. Using the primer *Eco_T7_YFP* as shown in sequence listing No. 6 and *Bam_SP6_YFP* as shown in sequence listing No. 7, the desired gene fragment was amplified by PCR and used analogously to the above description for preparing the dsRNA. The dsRNA YFP obtained is identical to the dsRNA used in use example 1 as non-sequence-specific control.

A dsRNA linked chemically at the 3' end of the RNA as shown in sequence listing No. 8 to the 5' end of the complementary RNA via a C18 linker group was prepared (L-dsRNA). To this end, synthons modified by disulfide bridges were used. The 3'-terminal synthon is bound to the solid support via the 3' carbon with an aliphatic linker group via a disulfide bridge. In the 5'-terminal synthon of the complementary oligoribonucleotide which is complementary to the 3'-terminal synthon of the one oligoribonucleotide, the 5'-trityl protecting group is bound via a further aliphatic linker and a disulfide bridge. Following synthesis of the two single strands, removal of the protecting groups and hybridization of the complementary oligoribonucleotides, the thiol groups which form are brought into spatial vicinity. The single strands are linked to each other by oxidation via their aliphatic linkers and a disulfide bridge. This is followed by purification with the aid of HPLC.

Preparation of the cell cultures:

The cells were incubated in DMEM supplemented with 4.5 g/l glucose, 10% fetal bovine serum in culture dishes at 37°C under a 7.5% CO₂ atmosphere and passaged before reaching confluence. The cells were detached with trypsin/EDTA. To prepare for microinjection, the cells were transferred into Petri dishes and incubated further until microcolonies formed.

- 18 -

Microinjection:

For the microinjection, the culture dishes were removed from the incubator for approximately 10 minutes. Approximately 50 nuclei were injected singly per
5 reaction within a marked area using the AIS microinjection system from Carl Zeiss, Göttingen, Germany. The cells were subsequently incubated for three more hours. For the microinjection, borosilicate glass capillaries from Hilgenberg GmbH, Malsfeld,
10 Germany, with a diameter of less than 0.5 μm at the tip were prepared. The microinjection was carried out using a micromanipulator from Narishige Scientific Instrument Lab., Tokyo, Japan. The injection time was 0.8 seconds and the pressure was approximately 100 hPa. The
15 transfection was carried out using the plasmid pCDNA YFP, which contains an approximately 800 bp *Bam*HI/*Eco*RI fragment with the YFP gene in vector pCDNA3. The samples injected into the nuclei contained 0.01 $\mu\text{g}/\mu\text{l}$ of pCDNA-YFP and Texas Red coupled to dextran-70000 in
20 14 mM NaCl, 3 mM KCl, 10 mM KPO_4 [sic], pH 7.5. Approximately 100 μl of RNA with a concentration of 1 μM or, in the case of the L-dsRNA, 375 μM were additionally added.

25 The cells were studied under a fluorescence microscope with excitation with the light of the excitation wavelength of Texas Red, 568 nm, or of YFP, 488 nm. Individual cells were documented by means of a digital camers. Figures 4a-e show the result for NIH3T3 cells.
30 In the cells shown in Fig. 4a, sense-YFP-ssRNA has been injected, in Fig. 4b antisense-YFP-ssRNA, in Fig. 4c dsRNA-YFP, in Fig. 4d no RNA and in Fig. 4e L-dsRNA.

The field on the left shows in each case the
35 fluorescence of cells with excitation at 568 nm. The fluorescence of the same cells at an excitation of 488 nm is seen on the right. The Texas Red fluorescence of all the cells shown demonstrates that the injection solution had been applied successfully into the nuclei

- 19 -

and that cells with successful hits were still alive after three hours. Dead cells no longer showed Texas Red fluorescence.

- 5 The right fields of each of figures 4a and 4b show that YFP expression was not visibly inhibited when the single-stranded RNA was injected into the nuclei. The right field of Fig. 4c shows cells whose YFP fluorescence was no longer detectable after the
- 10 injection of dsRNA-YFP. Fig. 4d shows cells into which no RNA had been injected, as control. The cell shown in fig. 4e shows YFP fluorescence which can no longer be detected owing to the injection of the L-dsRNA which shows regions with sequence homology to the YFP gene.
- 15 This result demonstrates that even shorter dsRNAs can be used for specifically inhibiting gene expression in mammals when the double strands are stabilized by chemically linking the single strands.

19a

Literature:

Asanuma, H., Ito, T., Yoshida, T., Liang, X. & Komiyama, M.
(1999). Photoregulation der Bildung und Dissoziation ei-
5 nes DNA-Duplexes durch *cis-trans*-Isomerisierung einer
Azobenzoleinheit. *Angew. Chem.* **111**, 2547-2549.

Azhayeva, E., Azhayev, A., Auriola, S., Tengvall, U., Urtti,
A. & Lönnberg, H. (1997). Inhibitory properties of double
10 helix forming circular oligonucleotides. *Nucl. Acids Res.*
25, 4954-4961.

Castelli, J., Wood, K.A. & Youle, R.J. (1998). The 2-5A system
in viral infection and apoptosis. *Biomed. Pharmacother.*
15 **52**, 386-390.

Dolinnaya, N.G., Blumenfeld, M., Merenkova, I., Oretskaya,
T.S., Krynetskaya, N.F., Ivanovskaya, M.G., Vasseur, M. &
Shabarova, Z.A. (1993). Oligonucleotide circularization
20 by template-directed chemical ligation. *Nucl. Acids Res.*
21, 5403-5407.

Expert-Bezancon, A., Milet, M. & Carbon, P. (1983). Precise
localization of several covalent RNA-RNA cross-link in
25 *Escherichia coli* 16S RNA. *Eur. J. Biochem.* **136**, 267-274.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E.
& Mello, C.C. (1998). Potent and specific genetic inter-
ference by double-stranded RNA in *Caenorhabditis elegans*.
30 *Nature* **391**, 806-811.

196

- Gao, H., Yang, M., Patel, R. & Cook, A.F. (1995). Circulaiza-
tion of oligonucleotides by disulfide bridge formation.
Nucl. Acids Res. **23**, 2025-2029.
- 5 Gryaznov, S.M. & Letsinger, R.L. (1993). Template controlled
coupling and recombination of oligonucleotide blocks con-
taining thiophosphoryl groups. *Nucl. Acids Res.* **21**, 1403-
1408.
- 10 Kaufman, R.J. (1999). Double-stranded RNA-activated protein
kinase mediates virus-induced apoptosis: A new role for
an old actor. *Proc. Natl. Acad. Sci. USA* **96**, 11693-11695.
- 15 Lipson, S.E. & Hearst, J.E. (1988). Psoralen cross-linking of
ribosomal RNA. In *Methods in Enzymology* Anonymous pp.
330-341.
- 20 Liu, Z.R., Sargueil, B. & Smith, C.W. (1998). Detection of a
novel ATP-dependent cross-linked protein at the 5' splice
site-U1 small nuclear RNA duplex by methylene blue-
mediated photo-cross-linking. *Mol. Cell. Biol.* **18**, 6910-
6920.
- 25 Micura, R. (1999). Cyclic oligoribonucleotides (RNA) by solid-
phase synthesis. *Chem. Eur. J.* **5**, 2077-2082.
- 30 Skripkin, E., Isel, C., Marquet, R., Ehresmann, B. & Ehres-
mann, C. (1996). Psoralen crosslinking between human im-
munodeficiency virus type 1 RNA and primer tRNA₃^{Lys}. *Nucl.*
Acids Res. **24**, 509-514.

19c

- Wang, S. & Kool, E.T. (1994). Circular RNA oligonucleotides. Synthesis, nucleic acid binding properties, and a comparison with circular DNAs. *Nucl. Acids Res.* **22**, 2326-2333.
- 5 Wang, Z. & Rana, T.M. (1996). RNA conformation in the Tat-TAR complex determined by site-specific photo-cross-linking. *Biochem.* **35**, 6491-6499.
- 10 Watkins, K.P. & Agabian, N. (1991). In vivo UV cross-linking of U snRNAs that participate in trypanosome trans-splicing. *Genes & Development* **5**, 1859-1869.
- 15 Wengel, J. (1999). Synthesis of 3'-C- and 4'-C-branched oligodeoxynucleotides and the development of locked nucleic acid (LNA). *Acc. Chem. Res.* **32**, 301-310.
- 20 Zwieb, C., Ross, A., Rinke, J., Meinke, M. & Brimacombe, R. (1978). Evidence for RNA-RNA cross-link formation in *Escherichia coli* ribosomes. *Nucl. Acids Res.* **5**, 2705-2720.

WO 00/44895

PCT/DE00/00244

1

Sequence Listing

<110> Kreutzer Dr., Roland
Limmer Dr., Stephan

<120> Method and medicament for inhibiting the
expression of a given gene

<130> 400968

<140>

<141>

<150> 199 03 713.2

<151> 1999-01-30

<150> 199 56 568.6

<151> 1999-11-24

<160> 8

<170> PatentIn Ver. 2.1

<210> 1

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of the artificial sequence:
EcoRI cleavage site, T7 RNA Polymerase
promoter

<400> 1

ggaattctaa tacgactcac tatagggcga tcagatctct agaag

45

<210> 2

WO 00/44895

PCT/DE00/00244

2

<211> 50
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of the artificial sequence:
 BamHI cleavage site, SP6 RNA Polymerase
 promoter

<400> 2
 gggatccatt taggtgacac tatagaatac ccatgatcgc gtagtcgata 50

<210> 3
 <211> 340
 <212> RNA
 <213> Artificial Sequence

<220>
 <223> Description of the artificial sequence:
 RNA which corresponds to a sequence from the
 positive control DNA of the HeLa Nuclear
 Extract in vitro transcription kit from
 Promega

<400> 3
 ucagaucucu agaagcuuua augcgguagu uuaucaacagu uaaaauugcua acgcagucag 60
 gcaccgugua ugaaaucuaa caaugcguc aucgucaucc ucggcacccgu caccucggau 120
 gcuguaggca uaggcuuggu uaugccgguu cugccggggc ucuugcggga uaucguccau 180
 uccgacagca ucgccaguca cuauggcgug cugcuagcgc uauaugcguu gaugcauuu 240
 cuaugcgcac ccguucucgg agcacugucc gaccgcuuug gccgccgcc aguccugcuc 300
 gcuucgcuaac uuggagccac uaucgacuac gcgaucaugg 340

<210> 4
 <211> 363
 <212> DNA
 <213> Artificial Sequence

<220>

WO 00/44895

PCT/DE00/00244

3

<223> Description of the artificial sequence:
 DNA which corresponds to a sequence from the
 positive control DNA of the HeLa Nuclear
 Extract in vitro transcription kit from
 Promega

<400> 4

```

tcagatctct agaagcttta atgcggtagt ttatcacagt taaattgcta acgcagtcag 60
gcaccgctga tgaatatctaa caatgcgctc atcgtcatcc tcggcaccgt caccctggat 120
gctgtaggca taggcttggg tatgccggtg ctgccggggc tcttgcgggg tategtccat 180
tccgacagca tcgccagtca ctatggcgtg ctgctagcgc tatatgcgtt gatgcaattc 240
ctatgcgcac ccgtttctcg agcactgtcc gaccgctttg gccgcgcgcc agtcctgtct 300
gcttcgctac ttggagccac tategcttac gcatcatgg cgaccacacc cgtcctgtgg 360
atc                                     363

```

<210> 5
 <211> 315
 <212> RNA
 <213> Artificial Sequence

<220>
 <223> Description of the artificial sequence:
 Sequence from the YFP gene

<400> 5

```

auggugagca agggcgagga gcugucacc gggguggugc ccauccuggu cgagcuggac 60
ggcgacguaa acggccacaa guucagcgug uccggcgagg gcgagggcga ugccaccuac 120
ggcaagcuga ccugaaaguu caucugcacc accggcaagc ugcccugucc cuggcccacc 180
cucgugacca ccugaccua cggcgugcag ugcuuacagc gcuaccccga ccacaugaag 240
cagcacgacu ucuucaaguc cgccaugccc gaaggcuacg uccaggagcg caccuucuu 300
uucaaggacg acggc                                     315

```

<210> 6
 <211> 52
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of the artificial sequence:

WO 00/44895

PCT/DE00/00244

4

EcoRI cleavage site, T7 RNA Polymerase promoter, complementary region to the YFP gene

<400> 6

ggattctaa tacgactcac tatagggcga atggtgagca agggcgagga gc 52

<210> 7

<211> 53

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of the artificial sequence:

BamHI cleavage site, SP6 RNA Polymerase promoter, complementary region to the YFP gene

<400> 7

gggatccatt taggtgacac tatagaatac gccgtcgtcc ttgaagaaga tgg 53

<210> 8

<211> 21

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of the artificial sequence:

RNA which corresponds to a sequence from the YFP gene

<400> 8

ucgagcugga cggcgacgua a 21

52032-2(S)

- 20 -

CLAIMS:

1. A method for inhibiting the expression of a given target gene in a mammalian cell, the method comprising introducing into the mammalian cell an oligoribonucleotide
5 with double-stranded structure (dsRNA) formed by two separate RNA single strands, said dsRNA having a length of 15 to 49 base pairs, wherein one of said RNA single strands has a region which is at least partially complementary to a given target gene expressed in a mammalian cell, wherein
10 said method is not practised on the human or animal body.
2. The method according to claim 1, wherein the dsRNA is enclosed by micellar structures.
3. The method according to claim 2, wherein the micellar structures are liposomes.
- 15 4. The method according to claim 1, wherein the dsRNA is enclosed by natural viral capsids or by chemically or enzymatically produced artificial capsids or structures derived therefrom.
5. The method according to any one of claims 1 to 4,
20 wherein the target gene is selected from the group consisting of an oncogene, a cytokine gene, an Id-protein gene, a development gene, and a prion gene.
6. The method according to any one of claims 1 to 4, wherein the target gene can be expressed in a pathogenic
25 organism.
7. The method according to claim 6, wherein the pathogenic organism is plasmodia.
8. The method according to any one of claims 1 to 4, wherein the target gene is part of a virus or viroid.

52032-2(S)

- 21 -

9. The method according to claim 8, wherein the virus or viroid is pathogenic for humans.

10. The method according to claim 8, wherein the virus or viroid is pathogenic for animals.

5 11. The method according to any one of claims 1 to 10, wherein the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

12. The method according to any one of claims 1 to 11,
10 wherein the cohesion of the RNA single strands, which is caused by hydrogen bonding between nucleotide pairs, is increased by at least one further chemical linkage.

13. The method according to any one of claims 1 to 11,
15 wherein cohesion of the RNA single strands, which is caused by hydrogen bonding between nucleotide pairs, is increased by at least two further chemical linkages.

14. The method according to claim 12 or 13, wherein the chemical linkage is formed by a covalent bond, an ionic bond, a hydrogen bond, hydrophobic interactions,
20 van-der-Waals interactions, stacking interactions, or by metal-ion coordination.

15. The method according to claim 14, wherein the chemical linkage is formed by van-der-Waals or stacking interactions.

25 16. The method according to any one of claims 12 to 15, wherein the chemical linkage is generated at at least one end of the dsRNA.

52032-2(S)

- 22 -

17. The method according to any one of claims 12 to 15, wherein the chemical linkage is generated at both ends of the dsRNA.

18. The method according to claim 12 or 13, wherein
5 the chemical linkage is formed by means of one or more linkage groups.

19. The method according to claim 18, wherein the linkage group is poly(oxyphosphinicooxy-1,3-propanediol), polyethylene glycol chains, or both poly(oxyphosphinicooxy-
10 1,3-propanediol) and polyethylene glycol chains.

20. The method according to claim 12 or 13, wherein the chemical linkage is formed by purine analogs used in the dsRNA in place of purines.

21. The method according to claim 12 or 13, wherein
15 the chemical linkage is formed by azabenzene units introduced into the dsRNA.

22. The method according to claim 12 or 13, wherein the chemical linkage is formed by branched nucleotide analogs used in the dsRNA in place of nucleotides.

20 23. The method according to claim 12 or 13, wherein at least one of the following groups is used for generating the chemical linkage: methylene blue; bifunctional groups, bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; or psoralene.

25 24. The method according to claim 12 or 13, wherein bis(2-chloroethyl)amine is used for generating the chemical linkage.

52032-2(S)

- 23 -

25. The method according to claim 12 or 13, wherein the chemical linkage is formed by thiophosphoryl groups provided at the ends of the dsRNA.

26. The method according to claim 12 or 13, wherein
5 the chemical linkage is formed by triple-helix bonds provided at the ends of the dsRNA.

27. The method according to any one of claims 1 to 26, wherein at least one 2'-hydroxyl group of the nucleotides of the dsRNA is replaced by a chemical group.

10 28. The method according to claim 27, wherein the chemical group is a 2'-amino or a 2'-methyl group.

29. The method according to any one of claims 1 to 28, wherein at least one nucleotide in at least one of said RNA single strands is a "locked nucleotide" with a sugar ring
15 which is chemically modified.

30. The method according to claim 29, wherein the nucleotide is chemically modified by a 2'-O, 4'-C-methylene bridge.

31. The method according to any one of claims 1 to 30,
20 wherein the dsRNA is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

32. The method according to any one of claims 1 to 31,
25 wherein one of the RNA single strands is complementary to the primary or processed RNA transcript of the target gene.

33. The method according to any one of claims 1 to 32, wherein the cell is a human cell.

52032-2(S)

- 24 -

34. The method according to any one of claims 1 to 33, wherein at least two dsRNAs as defined in claim 1 which differ from each other are introduced into the cell, and wherein at least segments of one strand of each dsRNA are
5 complementary to in each case one of at least two different target genes.

35. The method according to claim 34, wherein one of the target genes is the PKR gene.

36. Medicament for inhibiting the expression of a
10 given target gene in mammalian cells, said medicament comprising an oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands, said dsRNA having a length of 15 to 49 base pairs, wherein one of said RNA single strands has a region which is at
15 least partially complementary to a given target gene expressed in a mammalian cell.

37. The medicament according to claim 36, wherein the dsRNA is enclosed by micellar structures.

38. The medicament according to claim 37, wherein the
20 micellar structures are liposomes.

39. The medicament according to claim 36, wherein the dsRNA is enclosed by natural viral capsids or by chemically or enzymatically produced artificial capsids or structures derived therefrom.

25 40. The medicament according to claim 36, wherein the target gene is selected from the group consisting of an oncogene, a cytokine gene, an Id-protein gene, a development gene, and a prion gene.

52032-2(S)

- 25 -

41. The medicament according to any one of claims 36 to 39, wherein the target gene can be expressed in a pathogenic organism.

42. The medicament according to claim 41, wherein the
5 pathogenic organism is plasmodia.

43. The medicament according to any one of claims 36 to 39, wherein the target gene is part of a virus or viroid.

44. The medicament according to claim 43, wherein the
10 virus is a virus or viroid which is pathogenic for humans.

45. The medicament according to claim 43, wherein the virus or viroid is pathogenic for animals.

46. The medicament according to any one of claims 36 to 45, wherein the ends of the dsRNA are modified
15 in order to counteract degradation in the cell or dissociation into the single strands.

47. The medicament according to any one of claims 36 to 46, wherein the cohesion of the RNA single strands caused by hydrogen bonding between nucleotide pairs
20 is increased by at least one further chemical linkage.

48. The medicament according to any one of claims 36 to 46, wherein the cohesion of the RNA single strands caused by hydrogen bonding between nucleotide pairs is increased by two further chemical linkages.

25 49. The medicament according to claim 47 or 48, wherein the chemical linkage is formed by a covalent bond, an ionic bond, a hydrogen bond, hydrophobic interactions, van-der-Waals interactions, stacking interactions, or by metal-ion coordination.

52032-2(S)

- 26 -

50. The medicament according to claim 49, wherein the chemical linkage is formed by van-der-Waals interactions or stacking interactions.

51. The medicament according to any one of
5 claims 47 to 50, wherein the chemical linkage is generated at at least one end of the dsRNA.

52. The medicament according to claim 51, wherein the chemical linkage is generated at both ends of the dsRNA.

53. The medicament according to claim 47 or 48,
10 wherein the chemical linkage is formed by means of one or more linkage groups.

54. The medicament according to claim 53, wherein the linkage groups are poly(oxyphosphinicooxy-1,3-propanediol), polyethylene glycol chains, or both poly(oxyphosphinicooxy-
15 1,3-propanediol) and polyethylene glycol chains.

55. The medicament according to claim 47 or 48, wherein the chemical linkage is formed by purine analogs used in the dsRNA in place of purines.

56. The medicament according to claim 47 or 48,
20 wherein the chemical linkage is formed by azabenzene units inserted into the dsRNA.

57. The medicament according to claim 47 or 48, wherein the chemical linkage is formed by branched nucleotide analogs used in the dsRNA in place of
25 nucleotides.

58. The medicament according to claim 47 or 48, wherein at least one of the following groups is used for generating the chemical linkage: methylene blue;

52032-2(S)

- 27 -

bifunctional groups, bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; or psoralene.

59. The medicament according to claim 58, wherein bis(2-chloroethyl)amine is used for generating the chemical
5 linkage.

60. The medicament according to claim 47 or 48, wherein the chemical linkage is formed by thiophosphoryl groups provided at the ends of the dsRNA.

61. The medicament according to claim 47 or 48,
10 wherein the chemical linkage is formed by triple-helix bonds provided at the ends of the dsRNA.

62. The medicament according to any one of claims 36 to 61, wherein at least one 2'-hydroxyl group of the nucleotides of the dsRNA is replaced by a chemical
15 group.

63. The medicament according to claim 62, wherein the chemical group is a 2'-amino or a 2'-methyl group.

64. The medicament according to any one of claims 36 to 63, wherein at least one nucleotide in at least
20 one of the RNA single strands is a "locked nucleotide" with a sugar ring which is chemically modified.

65. The medicament according to claim 64, wherein the nucleotide is chemically modified by a 2'-O, 4'-C-methylene bridge.

25 66. The medicament according to any one of claims 36 to 65, wherein the dsRNA is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

52032-2(S)

- 28 -

67. The medicament according to any one of claims 36 to 66, wherein one of the RNA single strands is complementary to the primary or processed RNA transcript of the target gene.

5 68. The medicament according to any one of claims 36 to 67, wherein the cell is a human cell.

69. The medicament according to any one of claims 36 to 68, wherein at least two dsRNAs as defined in claim 36 which differ from each other are contained in the
10 medicament, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

70. The medicament according to claim 69, where one of the target genes is the PKR gene.

15 71. Use, for inhibiting the expression of a given target gene in mammalian cells, of an oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands, said dsRNA having a length of 15 to 49 base pairs, wherein one of said RNA single strands
20 has a region which is at least partially complementary to a given target gene expressed in a mammalian cell.

72. The use according to claim 71, wherein the dsRNA is enclosed by micellar structures.

73. The use according to claim 72, wherein the
25 micellar structures are liposomes.

74. The use according to claim 71, wherein the dsRNA is enclosed by natural viral capsids or by chemically or enzymatically produced artificial capsids or structures derived therefrom.

52032-2(S)

- 29 -

75. The use according to any one of claims 71 to 74, wherein the target gene is selected from the group consisting of an oncogene, a cytokine gene, an Id-protein gene, a development gene, and a prion gene.

5 76. The use according to any one of claims 71 to 75, wherein the target gene can be expressed in pathogenic organism.

77. The use according to claim 76, wherein the pathogenic organism is a plasmodia.

10 78. The use according to any one of claims 71 to 77, wherein the target gene is part of a virus or viroid.

79. The use according to claim 78, wherein the virus or viroid is pathogenic for humans.

80. The use according to claim 78, wherein the virus
15 or viroid is pathogenic for animals.

81. The use according to any one of claims 71 to 80, wherein the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

20 82. The use according to any one of claims 71 to 81, wherein the cohesion of the RNA single strands caused by hydrogen bonding between nucleotide pairs, is increased by at least one further chemical linkage.

83. The use according to claim 82, wherein the
25 cohesion caused by hydrogen bonding between nucleotide pairs is increased by two further chemical linkages.

84. The use according to claim 82 or 83, wherein the chemical linkage is formed by a covalent bond, an ionic bond, a hydrogen bond, hydrophobic interactions,

52032-2(S)

- 30 -

van-der-Waals interactions, stacking interactions, or by metal-ion coordination.

85. The use according to claim 84, wherein the chemical linkage is formed by van-der-Waals interactions or
5 stacking interactions.

86. The use according to any one of claims 82 to 85, wherein the chemical linkage is generated at at least one end of the dsRNA.

87. The use according to any one of claims 82 to 85,
10 wherein the chemical linkage is generated at both ends of the dsRNA.

88. Use according to claim 82 or 83, wherein the chemical linkage is formed by means of one or more linkage groups.

15 89. The use according to claim 88, wherein the linkage groups are poly(oxyphosphinicooxy-1,3-propanediol), polyethylene glycol chains, or both poly(oxyphosphinicooxy-1,3-propanediol) and polyethylene glycol chains.

90. The use according to claim 82 or 83, wherein the
20 chemical linkage is formed by purine analogs used in the dsRNA in place of purines.

91. The use according to claim 82 or 83, wherein the chemical linkage is formed by azabenzene units inserted into the dsRNA.

25 92. The use according to claim 82 or 83, wherein the chemical linkage is formed by branched nucleotide analogs used in the dsRNA in place of nucleotides.

93. The use according to claim 82 or 83, wherein at least one of the following groups is used for generating the

52032-2(S)

- 31 -

chemical linkage: methylene blue; bifunctional groups, bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; or psoralene.

94. The use according to claim 93, wherein

5 bis(2-chloroethyl)amine is used for generating the chemical linkage.

95. The use according to claim 82 or 83, wherein the chemical linkage is formed by thiophosphoryl groups attached to the ends of the dsRNA.

10 96. The use according to claim 82 or 83, wherein the chemical linkage is formed by triple-helix bonds provided at the ends of the dsRNA.

97. The use according to any one of claims 71 to 96, wherein at least one 2'-hydroxyl group of the nucleotides of
15 the dsRNA is replaced by a chemical group.

98. The use according to claim 97, wherein the chemical group is a 2'-amino or a 2'-methyl group.

99. The use according to any one of claims 71 to 97, wherein at least one nucleotide in at least one of the RNA
20 single strands is a "locked nucleotide" with a sugar ring which is chemically modified.

100. The use according to claim 99, wherein the nucleotide is chemically modified by a 2'-O, 4'-C-methylene bridge.

25 101. The use according to any one of claims 71 to 100, wherein the dsRNA is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

52032-2(S)

- 32 -

102. The use according to any one of claims 71 to 101, wherein one of the RNA single strands is complementary to the primary or processed RNA transcript of the target gene.

103. The use according to any one of claims 71 to 102,
5 wherein the cell is a human cell.

104. The use according to any one of claims 71 to 103, wherein at least two dsRNAs as defined in claim 71 which differ from each other are used, and wherein at least segments of one strand of each dsRNA are complementary to in
10 each case one of at least two different target genes.

105. The use according to claim 104, wherein one of the target genes is the PKR gene.

106. The use according to any one of claims 71 to 105, wherein the dsRNA is injectable into the bloodstream or into
15 the interstitium of the organism to undergo therapy.

107. An oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands, said dsRNA having a length of 15 to 49 base pairs, wherein one of said RNA single strands has a region which is at
20 least partially complementary to a given target gene expressed in a mammalian cell.

108. The oligoribonucleotide according to claim 107, wherein the dsRNA comprises a linker between the two RNA single strands.

25 109. The oligoribonucleotide according to claim 108, wherein the linker is a polyethylene glycol linker.

110. The oligoribonucleotide according to any one of claims 107 to 109, wherein said dsRNA is modified so as to be resistant to RNA degradation.

52032-2(S)

- 33 -

111. A composition comprising an oligoribonucleotide according to any one of claims 107 to 110, and a pharmaceutically acceptable carrier.

112. The composition according to claim 111, further comprising a second oligoribonucleotide according to any one of claims 107 to 110, wherein said second oligoribonucleotide differs in sequence from said oligoribonucleotide.

113. A mammalian cell comprising an exogenous oligoribonucleotide, wherein the exogenous oligoribonucleotide is an oligoribonucleotide according to any one of claims 107 to 110.

114. The mammalian cell according to claim 113, wherein the mammalian cell is a human cell.

115. The method according to any one of claims 1 to 35, wherein said dsRNA has a length of no more than 21 base pairs.

116. The medicament according to any one of claims 36 to 70, wherein said dsRNA has a length of no more than 21 base pairs.

117. The use according to any one of claims 71 to 106, wherein said dsRNA has a length of no more than 21 base pairs.

118. The oligoribonucleotide according to any one of claims 107 to 110, wherein said dsRNA has a length of no more than 21 base pairs.

119. The method according to any one of claims 1 to 35, wherein said dsRNA does not possess a 5' or 3' single-stranded overhang.

52032-2(S)

- 34 -

120. The medicament according to any one of claims 36 to 70, wherein said dsRNA does not possess a 5' or 3' single-stranded overhang.

121. The use according to any one of claims 71 to 106,
5 wherein said dsRNA does not possess a 5' or 3' single-stranded overhang.

122. The oligoribonucleotide according to any one of claims 107 to 110, wherein said dsRNA does not possess a 5' or 3' single-stranded overhang.

SMART & BIGGAR
OTTAWA, CANADA

PATENT AGENTS

WO 00/44895

PCT/DE00/00244

1/5

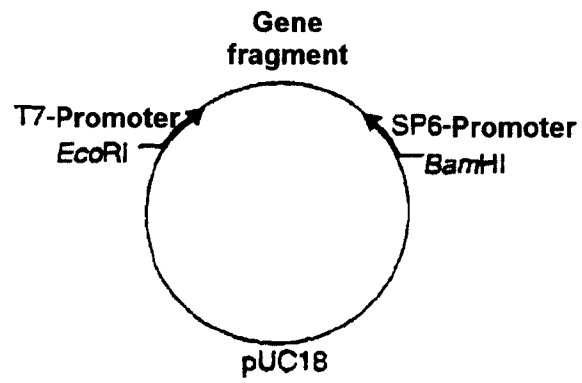


Fig. 1

Application number / numéro de demande: 2359180

Figures: 2, 4a, 4b, 4c, 4d, 4e

Pages: 3/5 4/5 5/5

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au
10^{ème} étage)

WO 00/44895

PCT/DE00/00244

3/5

1 2 3 4 5 6 7 8 9 10 11 12

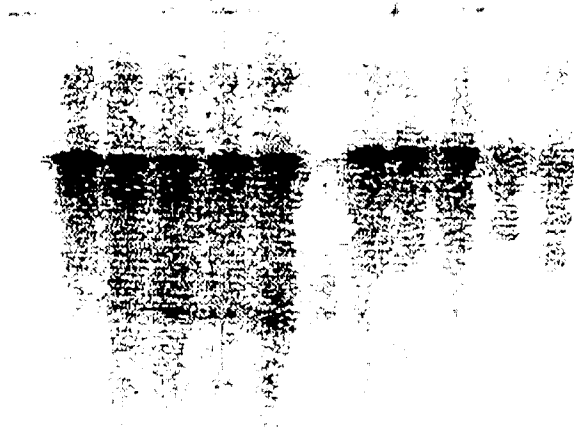


Fig. 3